1: AIDS Res Hum Retroviruses 1994 Jan;10(1):47-52

Retrovirus vector-mediated transfer of functional HIV-1 regulatory genes.

Garcia JV, Miller AD.

Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105.

Replication of the human immunodeficiency virus depends on the expression of its regulatory genes. We have constructed three plasmids, based on the retrovirus vector LXSN, that contain the tat, rev, and env (pLTRESN), the rev and env (pLRESN), and the nef (pLnefSN) genes of HIV-1. In a two-step virus rescue protocol, during which introns are removed from the DNA fragments inserted into pLXSN, these plasmids were used to establish amphotropic retrovirus vector producer lines for the transfer of tat (LtatSN), rev (LrevSN), and nef (LnefSN). These vectors have titers greater or equal to 10(6) CFU/ml and efficiently transduced each of these genes into a variety of human and murine cell lines. Representative populations of cells constitutively expressing the tat and rev genes were obtained. Cell lines transduced with LtatSN were able to trans-activate an HIV-LTRCAT construct, indicating the presence of a functional Tat protein. Similarly, cells transduced with LrevSN were able to rescue a rev- HIV-1 provirus, indicating the presence of a functional Rev. We also used LnefSN to obtain clones of cells expressing Nef. Our results indicate that these retrovirus vectors are useful reagents for the efficient transfer of functional Tat, Rev, and Nef and for the establishment of cell lines constitutively expressing these genes.

PMID: 8179963 [PubMed - indexed for MEDLINE]

i686-pc-linux-gnu Jun 12 2002 10:20:00

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 10, Number 1, 1994
Mary Ann Liebert, Inc., Publishers

Retrovirus Vector-Mediated Transfer of Functional HIV-1 Regulatory Genes

J. VICTOR GARCIA[1] and A. DUSTY MILLER[2]

ABSTRACT

Replication of the human immunodeficiency virus depends on the expression of its regulatory genes. We have constructed three plasmids, based on the retrovirus vector LXSN, that contain the *tat*, *rev*, and *env* (pLTRESN), the *rev* and *env* (pLRESN), and the *nef* (pLnefSN) genes of HIV-1. In a two-step virus rescue protocol, during which introns are removed from the DNA fragments inserted into pLXSN, these plasmids were used to establish amphotropic retrovirus vector producer lines for the transfer of *tat* (LtatSN), *rev* (LrevSN), and *nef* (LnefSN). These vectors have titers greater or equal to 10^6 CFU/ml and efficiently transduced each of these genes into a variety of human and murine cell lines. Representative populations of cells constitutively expressing the *tat* and *rev* genes were obtained. Cell lines transduced with LtatSN were able to trans-activate an HIV-LTRCAT construct, indicating the presence of a functional Tat protein. Similarly, cells transduced with LrevSN were able to rescue a *rev*$^-$ HIV-1 provirus, indicating the presence of a functional Rev. We also used LnefSN to obtain clones of cells expressing Nef. Our results indicate that these retrovirus vectors are useful reagents for the efficient transfer of functional Tat, Rev, and Nef and for the establishment of cell lines constitutively expressing these genes.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS (HIV) is a human retrovirus that causes acquired immune deficiency syndrome (AIDS).[1] Unlike murine retroviruses, HIV encodes a series of nonstructural proteins that regulate virus replication.[2] The nonstructural genes of HIV have been divided into two groups: regulatory and auxiliary genes.[3] The regulatory genes of HIV are *tat*, *rev*, and *nef*. Tat and Rev are necessary for viral replication and act on HIV via sequence-specific elements present in the viral RNA.[4–9] Tat increases the overall levels of all viral mRNAs and Rev promotes the transport of unspliced and singly spliced viral mRNAs from the nucleus to the cytoplasm.[10–16] Unlike Tat and Rev, Nef is not essential for virus propagation *in vitro* and its function in virus replication is controversial.[17–19]. Nef was reported to downregulate transcription via the viral long terminal repeat (LTR).[18,20,21] However, other investigators have reported that Nef has no influence on viral LTR transcription.[22,23] Most of the studies on these genes reported to date have used transfection as the means to express these genes *in vitro*. Although important information has been obtained by this technology, it has two significant disadvantages: (1) in most cases, multiple copies of plasmid are introduced at the same time into each cell, resulting in artificially high levels of the protein; and (2) most of the cell types used in AIDS research (e.g., lymphocytes) are difficult to transfect. Retroviral vectors serve as an efficient means to stably introduce foreign genes into a variety of target cells from multiple tissues both *in vivo* and *in vitro*.[24–26] To facilitate the study of these regulatory genes we have established and characterized retroviral producer lines for each of these genes. Here we describe the construction of these retrovirus producer lines and show that they can efficiently transduce these genes into a variety of cell types. Cells transduced by these retrovirus vectors express functional Tat, Rev, and Nef proteins. Our results show that these vectors promote efficient gene transfer and are useful to establish either individual cell clones or representative populations of cells constitutively expressing the regulatory genes of HIV.

[1]Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105.
[2]Program in Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104.

MATERIALS AND METHODS

Cells and culture conditions

Monolayer cell lines (NIH 3T3 TK$^-$, HeLa, PA317,[27] and PE501[28]) were grown in Dulbecco's modified Eagle medium (DMEM) with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin, and streptomycin at 37°C in a 10% CO_2 incubator.[27] Jurkat (obtained from D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle WA), HPBALL (obtained from E. Clark, University of Washington, Seattle, WA), Molt4, 8166 (obtained from M. Galatin, ICOS, Bothell, WA) SupT1, AA2, U937 (donated by J. Hoxie, M. Hershfield, and H. Koren and obtained from the NIH/AIDS Research and Reference Program), and CEM (ATCC) human cell lines were grown in RPMI-1640 medium supplemented with 12.5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10% fetal bovine serum, penicillin, streptomycin, and amphotericin B (Fungizone) at 37°C in a 5% CO_2 incubator.

Retrovirus vector construction

All HIV fragments were cloned into the retroviral vector pLXSN,[28] using standard techniques as follows: pLTRESN was constructed by ligating an *Eco*RI–*Xho*I fragment (nucleotides 5750–8914) from the HIV-1 isolate SF2 (obtained from P. Luciw, University of California, Davis)[29] into the *Eco*RI and *Xho*I restriction sites of pLXSN. This 3164-bp fragment contains the *tat*, *rev*, and *env* genes of HIV. pLRESN was constructed by removing 194 bp of the first *tat* exon from pLTRESN by digestion with *Eco*RI and *Mlu*I and religation. pLnefSN has been described[30] and was constructed by ligation of a *Bg*lII fragment (nucleotides 8504–9574) from HIV-1 SF2 into the *Bam*HI restriction site in pLXSN.

Generation of stable vector-producing cell lines

Stable retrovirus vector-producing cell lines were generated by calcium phosphate transfection, essentially as previously described.[28] Briefly, PE501 ecotropic packaging cells were tranfected with 10 μg of plasmid DNA. Ecotropic virus harvested from the PE501 cells was used to infect PA317 cells. Individual cell clones (5-15) selected in medium containing G418 (1.5 mg/ml, 50% active; GIBCO, Grand Island, NY) were isolated from plates with a small number of colonies, using glass cloning rings. Individually isolated clones were assayed for virus titer and for the structure of the integrated provirus (see below). Virus titer was determined by infection of NIH 3T3 TK$^-$ cells (5×10^5) with 1–5 μl of the filtered PA317 conditioned medium as described above. Infected cells were split (1:10 and 1:100) and cultured in medium containing G418 (1.5 mg/ml). Five to 7 days later cell colonies were stained with Coomassie blue and counted.

Retroviral gene transfer into human cells

HeLa cells were infected as described above for NIH 3T3 TK$^-$ cells, except that selection was carried out in G418 (2 mg/ml; 50% active) for 7 to 10 days. CEM, Jurkat, Molt4, 8166, SupT1, HPBALL, U937, and AA2 cells ($0.5–1 \times 10^6$) were incubated overnight with the appropriate virus supernatant (0.1 to 2.5 ml) in medium (4 ml) containing Polybrene (4 μg/ml). Virus and Polybrene were removed by centrifugation and cells plated in fresh medium (4 ml) containing G418 (1.5–2 mg/ml; 50% active). Ten to 14 days later cells were transferred to 10-cm dishes and 6 ml of fresh medium with G418 was added. Five to 7 days later cells were split (1:20 or 1:10) into fresh medium containing G418. Cultures were then passage as necessary in medium without G418.

DNA and RNA analysis

Genomic DNA was prepared from individual PA317 producer clones and analyzed for provirus structure by Southern blotting after digestion with *Xba*I, which cuts once in both LTRs as described.[25,26] RNA was prepared by the guanidium thiocyanate method[31] and analyzed by Northern blotting as described.[26] DNA and RNA blots were probed with a ^{32}P-labeled *neo*-specific DNA probe.

Analysis of Tat, Rev, and Nef expression

Expression of functional Tat in adherent cells was determined by measuring trans-activation of a transfected HIV-LTR-CAT construct (pH3LTRCAT, provided by I. Chen, University of California, Los Angeles). Tat expression in human T and B cell lines was determined by measuring trans-activation of the pH3LTRCAT construct after electroporation as follows: suspension cells (1×10^7) were collected by centrifugation and grown in 25 ml of medium in a T75 flask for 2 days. Electroporation and CAT assay conditions were similar to those described by Cann *et al.*,[32] except that 200 V was used. Rev expression was monitored by rescue of p24 production by a rev$^-$ HIV-1 provirus (JR-CSF delta-Sal; provided by S. Arrigo and I. Chen, University of California, Los Angeles)[33] after transfection (monolayer cells) or electroporation (T and B cells) as described above. Particle-associated p24 was determined with an antigen trap assay kit (Coulter [Hialeah, FL] or Abbott [Abbott Park, IL]), following manufacturer instructions. Nef expression was determined by immunoprecipitation and Western blot analysis after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a rabbit antiserum against recombinant Nef produced in bacteria (obtained from L. Ratner, Washington University, St. Louis, MO). Briefly, cells were lysed in radioimmunoprecipitation (RIPA) buffer containing protease inhibitors and 1 μl of the anti-Nef antiserum was added. Samples were incubated on ice for 1 hr and immunocomplexes were separated by binding to protein A–Sepharose (Pharmacia, Piscataway, NJ). Samples were then separated by SDS-PAGE on a 12% gel, transferred to nitrocellulose, and probed with the anti-Nef antiserum (1:500 dilution). Blots were developed by using an alkaline phosphatase labeled goat anti-rabbit antibody (Promega, Madison, WI).

RESULTS

Characterization of retrovirus producer lines

The strategy followed to make the retroviral producer cell lines involved the transient rescue of virus from an ecotropic

retrovirus packaging cell line, which in turn was used to infect an amphotropic packaging line. This general approach yields stable vector-producing cell lines containing single integrated proviruses. At the same time, introns present in the inserted fragments are removed during the single round of retrovirus replication (Fig. 1). In the case of pLTRESN, six PA317 clones were analyzed. Four had the correct (e.g., spliced) proviral insert, and two had rearranged provirus inserts (data not shown). The resultant LtatSN producer lines had titers ranging from 4×10^5 to 3×10^6 CFU/ml. Clone 2B4 had the highest titer (3×10^6 CFU/ml) and was used for the experiments described below. To make the Rev retrovirus producer line, a 194-bp fragment (*Eco*RI to *Mlu*I) of the first exon of *tat* was removed from pLTRESN to generate pLRESN. Following the same strategy described (Fig. 1), 10 independent PA317 clones were analyzed for proviral structure and titer. Nine of the proviruses were approximately 3400 bp in length (Fig. 2A), indicative of a provirus that has deleted the intron containing most of the HIV *env* gene. This was further confirmed at the RNA level by Northern blot analysis (Fig. 2B). The titers of the PA317/LrevSN producer lines ranged between 10^3 and 10^6 CFU/ml. Clone NA3 had the highest titer (1×10^6 CFU/ml) and was used in all subsequent experiments. The PA317/LnefSN producer line was prepared in the same way and virus titers ranged between 6×10^4 and 1×10^6. Clone 11 had a titer of 10^6 CFU/ml and was used in all experiments.

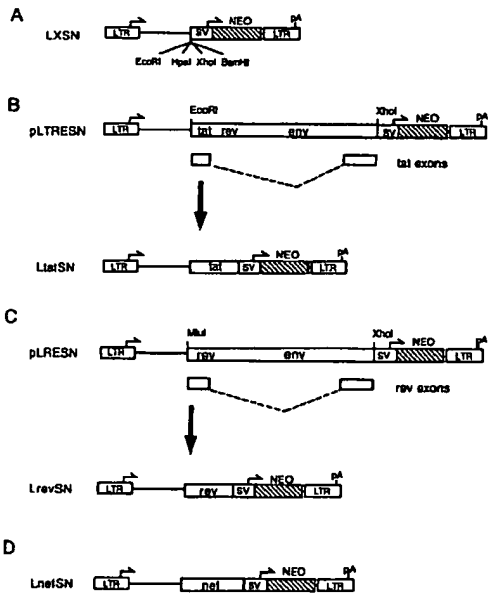

FIG. 1. Retroviral vector construction. (A) pLXSN[28] was the parent vector into which HIV fragments were inserted. In all cases, HIV sequences were driven by the Moloney murine leukemia virus long terminal repeat (LTR). The selectable marker (*neo*) is driven by the simian virus 40 (SV40) promoter. (B) Tat-expressing vector before (top) and after (bottom) introns are removed. (C) Rev-expressing vector before (top) and after (bottom) introns are removed. (D) Nef-expressing vector.

HIV long terminal repeat trans-activation by Tat-expressing cell lines

To determine if LtatSN produced virus containing a functional *tat* gene, U937, AA2, HPBALL, SupT1, HeLa, and NIH 3T3 TK$^-$ cells were transduced with LtatSN. After selection in G418, representative populations of cells were tested for Tat expression using a functional assay based on HIV LTR trans-activation. pH3LTRCAT, a plasmid that contains the *cat* gene under the control of the HIV LTR, was transfected (adherent cell lines) or electroporated (suspension cell lines)[32] into cells transduced with LtatSN or control viruses. These results show that retroviral-mediated gene transfer by itself does not affect the basal level of LTR trans-activation. Only in cells transduced with LtatSN is the LTR efficiently trans-activated. Extracts from all cell lines transduced with LtatSN showed markedly higher CAT activities than corresponding cells transduced with a control vector that expressed only *neo* (Fig. 3A and B) or with the *rev* vector (Fig. 3B).

HIV rev$^-$ mutant complementation by Rev-expressing cell lines

To monitor Rev expression in cells transduced with LrevSN, the *rev*$^-$ mutant complementation assay described by Arrigo *et al.* was employed.[33] In the absence of Rev, RNA transcripts coding for the *gag*, *pol*, and *env* genes are retained in the nucleus and not translated into proteins.[12,14,33] In this assay a *rev*$^-$ mutant of JR-CSF (delta-Sal) was introduced into human T, B, and monocytic/macrophage cell lines and HeLa cells transduced with LrevSN or with a control virus. Only cells transduced with LrevSN complemented the *rev*$^-$ provirus as determined by the accumulation of a significant amount of p24 in the culture supernatant (Table 1). Although most control cell lines produced a low level of p24, the only two control non-T cell lines tested, AA2 and HeLa, had 3–30 times more background p24, suggesting a small level of Rev-independent RNA leakage to the cytoplasm in these cells. These results indicate that LrevSN can transduce a functional *rev* gene able to fully complement a *rev*$^-$ HIV-1 provirus.

Analysis of Nef expression after retroviral-mediated gene transfer

Two independent groups have reported difficulty in establishing cell lines constitutively expressing Nef, raising the possibility that Nef expression might be toxic.[17,34] Using retrovirus-mediated gene transfer, we have shown that populations of human cells constitutively expressing functional Nef can be obtained.[30] However, these populations of cells have been established under conditions that favor integration of a single provirus per cell. They also were maintained in culture for 2–3 weeks prior to analysis for Nef expression.[30] To determine the efficiency of *nef* transfer, we transduced NIH 3T3 TK$^-$ cells with LnefSN. We then isolated individual G418-resistant clones and determined Nef expression levels. Extracts from four of the five clones analyzed showed similar levels of a protein of ~29 kDa that reacted with Nef antiserum (Fig. 4). The fact that four of five individual clones analyzed express Nef indicates that transduction of the *nef* gene is efficient and further suggests that Nef expression per se is not deleterious to these cells.

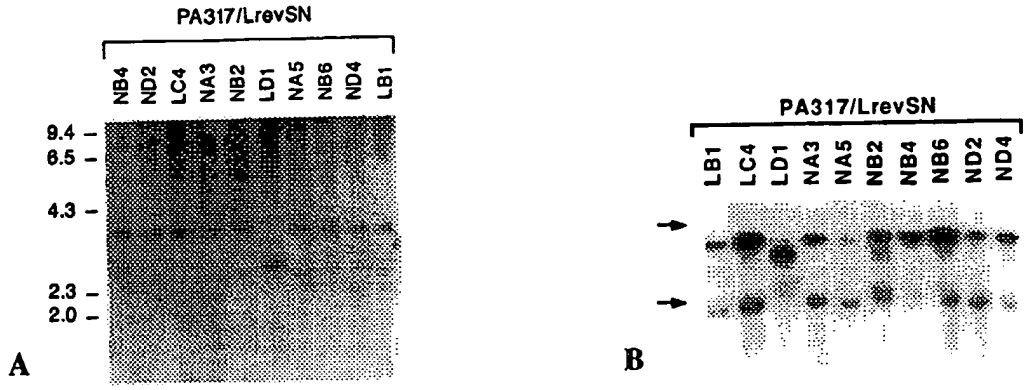

FIG. 2. Proviral structure of the LrevSN producer lines. (A) Southern blot analysis of LrevSN amphotropic producer lines. The band that migrates at approximately 3400 bp corresponds to LrevSN. Molecular weight markers are indicated on the left (kbp). (B) Northern blot analysis of LrevSN amphotropic producer lines. The high molecular weight band corresponds to the viral RNA encoding the *rev* gene, the lower molecular weight band corresponds to the SV40-driven *neo* gene. Arrows indicate the position in the gel of the ribosomal RNA (28S and 18S). Both blots were probed with a *neo*-specific DNA probe.

DISCUSSION

Efficient replication of HIV is dependent on the expression of its regulatory genes.[2,3] To further advance our understanding of how these genes affect both HIV replication and host cell gene expression, a series of plasmids based on the retrovirus vector pLXSN were constructed that contain the *tat*, *rev*, *env*, and *nef* genes of HIV-1. These vectors were used to obtain amphotropic retrovirus vector producer lines that efficiently transduced each of the three HIV regulatory genes. The producer cell lines described represent a significant advance over those previously reported in that they have significantly higher titers, and only one HIV gene is transferred in each case.[35,36] A retrovirus vector similar to pLTRESN containing the HIV *tat*, *rev*, and *env* genes in a single construct was described as capable of transducing all three genes.[36] Under our experimental conditions, and with our vector, only the *tat* gene is efficiently transferred. In the case of the vector containing the *rev* and *env* genes, pLRESN, it was surprising to note that this construct was able to efficiently transduce only the *rev* gene and that all of the clones analyzed had deleted most of the *env* gene. Because this construct expresses functional Rev, it might have been expected that at least some of the clones isolated would contain both *rev* and *env* genes. It is unclear at this point why we did not obtain producer lines expressing both Env and Rev. However, it is possible that under our experimental conditions Rev might not be fully functional in the mouse PE501 cell line as described by other investigators in rodent cell lines.[37,38]

All cells infected with LtatSN were able to trans-activate the HIV LTR, demonstrating that the transduced *tat* gene was fully functional. Sequences in human chromosome 12 have been described as required for trans-activation of the HIV LTR in

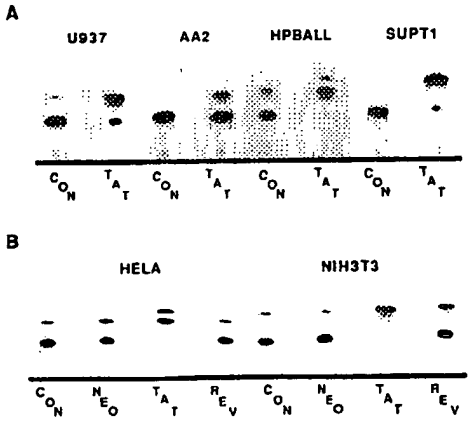

FIG. 3. HIV LTR trans-activation in cells transduced with LtatSN. (A) Functional Tat expression in human monocytic/macrophage (U937), B (AA2), and T (HPBALL, SupT1) cell lines transduced with LtatSN (tat) or a control vector (con). (B) HIV LTR trans-activation in human Hela and mouse NIH 3T3 TK$^-$ cell lines (con), cells transduced with a control vector (*neo*), with LtatSN (*tat*), or with LrevSN (*rev*). The presence of functional Tat was determined by measuring trans-activation of an LTR-CAT construct as described in Methods. The lower spot corresponds to the [^{14}C]chloramphenicol. The top spots correspond to the acetylated forms of [^{14}C]chloramphenicol.

TABLE 1. COMPLEMENTATION OF *rev*$^-$ MUTANT OF HIV BY LrevSN[a]

	HIV p24 production (pg/ml)	
Cell line	*Control cells*	*LrevSN-transduced cells*
CEM	50[b]	1500
Jurkat	5	3045
Molt4	20	1335
SupT1	40	3540
AA2	190	3925
HeLa	156	2106

[a]Rev function was assayed by measuring p24 produced by a *rev*$^-$ HIV-1 provirus introduced into the different cell lines by electroporation or transfection as described.[33]

[b]p24 (pg/ml) was measured with a commercial ELISA kit (see Materials and Methods).

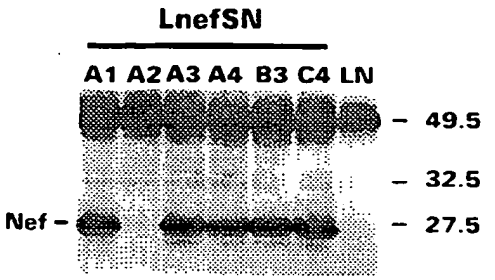

FIG. 4. Nef expression in cells infected with LnefSN. Extracts from NIH 3T3 TK$^-$ cell clones transduced with LnefSN or a control vector (LN) were immunoprecipitated with anti-Nef rabbit antiserum and separated on an SDS-PAGE gel as described in Materials and Methods. Samples were then blotted onto nitrocellulose and probed with Nef antiserum. The blot was developed with an alkaline phosphatase-labeled goat anti-rabbit (Fc-specific) antibody. The position of the Nef protein in the gel is indicated. Migration of prestained molecular weight markers (kDa) is indicated on the right. The dark band that migrates above the 49.5-kDa marker is the Fc portion of the IgG used in the immunoprecipitation prior to Western blot.

CHO cells.[39] Under our experimental conditions mouse NIH 3T3 TK$^-$ cells transduced with LtatSN efficiently trans-activated the HIV LTR in the absence of human DNA sequences. These results indicate that NIH 3T3 TK$^-$ cells contain all factors required for *tat* function. Cells transduced with LrevSN expressed functional Rev as determined by its ability to complement an HIV *rev*$^-$ mutant. Of all the cell lines tested, AA2 and HeLa had the highest level of background p24 produced by the HIV *rev*$^-$ mutant. The molecular basis of this leakage is at present not understood but it might indicate the presence of a cellular factor(s) that partially complement the function of Rev. Transduction of *nef* was efficiently done by LnefSN, using standard methodology.

The ability of the retrovirus vectors described here to efficiently transfer the regulatory genes of HIV will be helpful in studying their effects on cell function was well as gene expression in representative cell populations. Because the number of vector copies integrated into the genome is low when compared to transfected populations, more homogeneous levels of expression are usually obtained, resulting in more meaningful observations. Retrovirus vectors have been used to express foreign genes *in vivo* via bone marrow transplantation or by direct injection into liver in mice.[25,40] Therefore the vectors described here should also be useful in the development of small animal models for the study of the role of Tat, Rev, and Nef in HIV pathogenesis and disease progression. Cell lines that constitutively express Tat and Rev will be useful to produce stocks of infectious viruses lacking either functional Tat or Rev by trans-complementation.[41] The use of cell lines that contain a single integrated copy of *tat* or *rev* will minimize the chance of recombinations that could produce replication-competent virus. This in turn will be useful in addressing their role in HIV latency. The availability of an efficient retrovirus vector for *nef* transfer has already proved to be useful in addressing the effect of Nef on CD4 surface levels.[30,42,43] More recently a similar vector has also been useful in assessing the effect of SIV Nef on human CD4 as well.[44] In the future, LnefSN should be helpful in addressing the mechanism of CD4 downmodulation by Nef.

ACKNOWLEDGMENTS

We thank J. Kimpton and A. Frazier for technical assistance, Dr. P. Luciw for the p9B18 plasmid, Dr. Lee Ratner for the Nef antiserum, Drs. D. Geraghty, E. Clark, and M. Galatin for cell lines, Drs. S. Arrigo, A. Cann, J. Zack, W. O'Brian, P. Green, and I. Chen for the delta-Sal and pH3LTRCAT plasmids, their advice, and their support during the early phases of this work, and Drs. R. V. Srinivas, R. M. Goorha, and S. Anderson for critically reviewing this manuscript. The following reagents were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: AA2 from M. Hershfield, SupT1 from J. Hoxie, and U937 from H. Koren. This work was funded in part by Grant AI27291 from the NIAID to A.D.M., Grant CA59175 to J.V.G., CORE Grant CA-21765, and the American Lebanese Syrian Associated Charities.

REFERENCES

1. Fauci AS: The human immunodeficiency virus: Infectivity and mechanisms of pathogenesis. Science 1988;230:617–622.
2. Cullen BR: Regulation of human immunodeficiency virus replication. Annu Rev Microbiol 1991;45:219–250.
3. Vaishnav YN and Wong-Staal F: The biochemistry of AIDS. Annu Rev Biochem 1991;60:577–630.
4. Dayton AI, Sodroski JG, Rosen CA, Goh WC, and Haseltine WA: The trans-activator gene of the human T-cell lymphotrophic virus type III is required for replication. Cell 1986;44:941–947.
5. Fisher AG, Feinberg MB, Josephs SF, Harper ME, Marselle LM, Reyes G, Gonada MA, Aldovini A, Debouk C, Gallo RC, and Wong-Staal F: The trans-activator gene of HTLV-III is essential for virus replication. Nature (London) 1986;320:367–371.
6. Rosen CA, Sodroski JG, and Haseltine WA: The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell 1985;41:813–823.
7. Sodroski J, Goh WC, Rosen C, Dayton A, Terwilliger E, and Haseltine WA: A second post-transcriptional trans-activator gene required for the HTLV-III replication. Nature (London) 1986;321:412–417.
8. Feng S and Holland EC: HIV-1 *tat* trans-activation requires the loop sequence within TAR. Nature (London) 1988;334:165–167.
9. Garcia JA, Harrich D, Soultanakis E, Wu F, Mitsuyasu R, and Gaynor RB: Human immunodeficiency virus type 1: LTR TATA and TAR region sequences required for transcriptional regulation. EMBO J 1989;8:765–778.
10. Selby MJ, Bain ES, Luciw PA, and Peterlin BM: Structure, sequence, and position of the stem-loop structure in tar determine transcriptional elongation by tat through the HIV-1 long terminal repeat. Genes Dev 1989;3:547–558.
11. Chang D and Sharp P: Regulation by HIV Rev depends upon recognition of splice sites. Cell 1989;59:789–795.
12. Feinberg MB, Jarret RF, Aldovini A, Gallo RC, and Wong-Staal F: HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell 1986;46:807–817.

13. Felber BK, Hadzopoulou-Cladaras M, Cladaras C, Copeland T, and Pavlakis GN: The Rev protein of HIV-1 affects the stability and transport of the viral mRNA. Proc Natl Acad Sci USA 1989;86:1495–1499.
14. Emerman M, Vazeux R, and Peden K: The *rev* gene product of the human immunodeficiency virus affects envelope-scientific RNA localization. Cell 1989;57:1155–1165.
15. Hammarskjold ML, Heimer J, Hammarskjold B, Sangwan I, Albert L, and Rekosh D: Regulation of human immunodeficiency virus env expression by the rev gene product. J Virol 1989;63:1959–1966.
16. Malim MH, Hauber J, Le S, Maizel JV, and Cullen BR: The HIV-1 Rev transactivator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) 1989;338:254–257.
17. Cheng-Mayer C, Iannello P, Shaw K, Luciw PA, and Levy JA: Differential effects of nef on HIV replication: Implications for viral pathogenesis in the host. Science 1989;246:1629–1632.
18. Niederman MJ, Thielan BJ, and Ratner L: Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc Natl Acad Sci USA 1989;86:1128–1132.
19. Luciw PA, Cheng-Mayer C, and Levy JA: Mutational analysis of the human immunodeficiency virus: The orf-B region down-regulates virus replication. Proc Natl Acad Sci USA 1987;84:1434–1438.
20. Terwilliger E, Sodroski JG, Rosen CA, and Haseltine WA: Effects of mutations within the 3′ orf open reading frame region of human T-cell lymphotropic virus type III (HTLV-III/LAV) on replication and cytopathogenicity. J Virol 1986;60:754–760.
21. Ahmad N and Venkatesan S: Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science 1988;241:1481–1485.
22. Hammes S, Dixon E, Malim M, Cullen B, and Greene W: Nef protein of human immunodeficiency virus type 1: Evidence against its role as a transcriptional inhibitor. Proc Natl Acad Sci USA 1989;86:9549–9553.
23. Kim S, Ikeuchi K, Byrn R, Groopman J, and Baltimore D: Lack of a negative influence on viral growth by the *nef* gene of human immunodeficiency virus type 1. Proc Natl Acad Sci USA 1989; 86:1–5.
24. Hock RA, Miller AD, and Osborne WRA: Expression of human adenosine deaminase from various strong promoters after gene transfer into human hematopoietic cell lines. Blood 1989;74:876–881.
25. Kaleko M, Garcia JV, and Miller AD: Persistent gene expression after retroviral gene transfer into liver cells in vivo. Hum Gene Ther 1991;2:27–32.
26. Palmer TD, Hock RA, Osborne WRA, and Miller AD: Efficient retrovirus-mediated transfer and expression of a human adenosine deaminase gene in diploid skin fibroblasts from an adenosine deaminase-deficient human. Proc Natl Acad Sci USA 1987; 84:1055–1059.
27. Miller AD and Buttimore C: Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol 1986;6:2895–2902.
28. Miller AD and Rosman GJ: Improved retroviral vectors for gene transfer and expression. Biotechniques 1989;7:980–990.
29. Sanchez-Pescador R, Power MD, Barr PJ, Steimer KS, Stempien MM, Brown-Shimer SL, Gee WW, Renard A, Randolph A, Levy JA, Dina D, and Luciw PA: Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). Science 1985;227:484–492.
30. Garcia JV and Miller AD: Serine phosphorylation-independent downregulation of cell-surface CD4 by Nef. Nature (London) 1991;350:508–511.
31. Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–159.
32. Cann AJ, Koyanagi Y, and Chen ISY: High efficiency transfection of primary human lymphocytes and studies of gene expression. Oncogene 1989;3:123–128.
33. Arrigo SJ, Weitsman S, Rosenblatt JD, and Chen ISY: Analysis of *rev* gene function on human immunodeficiency virus type 1 replication in lymphoid cells by using a quantitative polymerase chain reaction method. J Virol 1989;63:4875–4881.
34. Luria S, Chambers I, and Berg P: Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. Proc Natl Acad Sci USA 1991;88:5326–5330.
35. Koka P, Yunis J, Passarelli AL, Dubey DP, Faller DV, and Yunis EJ: Increased expression of CD4 molecules on Jurkat cells mediated by human immunodeficiency virus Tat protein. J Virol 1988; 62:4353–4357.
36. Stevenson M, Meier C, Mann AM, Chapman N, and Wasiak A: Envelope glycoprotein of HIV induces interference and cytolysis resistance in $CD4^+$ cells: Mechanism for persistence in AIDS. Cell 1988;53:483–496.
37. Trono D and Baltimore D: A human cell factor is essential for HIV-1 Rev action. EMBO J 1990;9(12):4155–4160.
38. Winslow BJ and Trono D: The blocks to human immunodeficiency virus type 1 Tat and Rev functions in mouse cell lines are independent. J Virol 1993;67(4):2349–2354.
39. Hart CE, Ou C-Y, Galphin JC, Moore J, Bacheler LT, Wasmuth JJ, Petteway SR Jr, and Schochetman G: Human chromosome 12 is required for elevated HIV-1 expression in human-hamster hybrid cells. Science 1989;246:488–491.
40. Kaleko M, Garcia JV, Osborne WRA, and Miller AD: Expression of human adenosine deaminase in mice after transplantation of genetically-modified bone marrow. Blood 1990;75:1733–1741.
41. Chen H, Boyle TJ, Malim MH, Cullen BR, and Lyerly HK: Derivation of a biologically contained replication system for human immunodeficiency virus type 1. Proc Natl Acad Sci USA 1992; 89:7678–7682.
42. Garcia JV, Alfano J, and Miller AD: The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. J Virol 1993;67:1511–1516.
43. Anderson S, Shugars DC, Swanstrom R, and Garcia JV: Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. J Virol 1993;67:4923–4931.
44. Benson RE, Sanfridson A, Ottinger JS, Doyle C, and Cullen BR: Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. J Exp Med 1993;177:1561–1566.

Address reprint requests to:
J. Victor Garcia
Department of Virology and Molecular Biology
St. Jude Children's Research Hospital
332 N. Lauderdale
Memphis, Tennessee 38105